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博 士 論 文 概 要

論 文 題 目

Functional analysis of the Polycomb group ESC-E(Z) complex using medaka as a model system

メダカをモデルシステムとして用いた
ポリコーム遺伝子群
ESC-E(Z)**複合体の機能解析**

申 請 者

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Generation of a multicellular organisms from a fertilized egg requires the establishment and maintenance of multiple cell lineages with specific differentiated traits. The establishment of cell lineages, each committed to a defined differentiation pathway, is dependent upon epigenetic processes that regulate expression patterns of the appropriate genes. Maintenance of the established lineages requires stable inheritance of these patterns across multiple cell divisions during development. This phenomenon has been termed cellular memory, and is thought to involve heritable changes in chromatin structure. Genetic studies in *Drosophila* have shown that *Polycomb* group (PcG) is involved in this process. In this study, I performed functional analysis of PcG genes using medaka fish as a model system. Among the PcG genes, I focused on the particular members, the protein products of which constitute the ESC-E(Z) complex.

In the first chapter, I gave an overview about the current circumstances of cellular memory research and clarify the importance of PcG genes for this process with special emphasis on ESC-E(Z) complex. I also refer to the advantage of using medaka as a model system.

In the 2nd chapter, I described the isolation and expression profile of medaka homologs of *extra sex combs* (*oleed*) and *Enhancer of zeste* (*olezh2*), the putative members of medaka ESC-E(Z) complex. Both genes were highly conserved especially among vertebrates. The temporal expression profile was examined by Northern blots using total RNA extracted from successive embryonic stages. As expected, the profiles of the temporal expression of these genes were overlapping but not identical, suggesting the existence of the variable ESC-E(Z) complexes in development. The spatial distributions, monitored by whole-mount *in situ* hybridization, were nearly identical, suggesting the proteins encoded function together during development.

In the 3rd chapter, I described the knock-down analysis of both genes, which resulted in cyclopia phnotype. In mouse, mutations of *eed*, the *esc* homolog, and targeted disruption of *Ezh2*, the *E*(*z*) homolog, resulted in embryonic lethality, making it difficult to understand their role in development. To circumvent this situation, I took advantage of morpholino antisense oligonucleotides (MOs), which have been shown to be effective and specific translational inhibitors in several species. Microinjection of MO led to the inhibition of target protein in a dose-dependent manner. This enables me to perform functional analysis under hypomorphic conditions, referred to as knock-down experiments. Inhibition of the protein synthesis by injection of a higher amount of MOs resulted in total lethality as early as gastrula stage. Therefore, I injected a smaller amount of MO to generate hypomorphic conditions. Under these conditions, overall lethality was reduced to below 10% and the surviving morphants (a term for MO-injected embryos) displayed various phenotypes such as cyclopia, developmental retardation, increased cell death, abnormal ear patterning, short body length, and defects in blood circulation. Because cyclopia was consistently found and the most prominent phenotype, I focused on this phenotype and performed more detailed studies.

In the 4th chapter, I described that the cyclopia phenotype is caused by impairment of ESC-E(Z) complex. The amounts of MOs of *olezh2* as well as *oleed* were reduced until the frequency of the normal embryos reached more than 70%. Coinjection of the both MOs at these doses resulted in the cyclopia phenotype with a high frequency (more than 70%), suggesting the cooperation between these two genes. Additionally, inhibition of histone deacetylase (HDAC), the tentative component of the ESC-E(Z) complex, with trichostatin A (TSA) also resulted in

cyclopia phenotype.

In the 5th chapter, I described how the impairment of ESC-E(Z) complex resulted in cyclopia. This chapter is subdivided into two parts. The cyclopia phenotype has been implicated frequently in defects in nodal or *hedgehog* signaling pathway. So, in the first part, I examined whether the nodal signaling is impaired in embryos defective in the ESC-E(Z) function. Most of the nodal mutant embryos that exhibit severe cyclopia phenotype have strict defects in prechordal plate formation. The *goosecoid* (*gsc*) is a homeobox-containing gene, ubiquitously found in a variety of vertebrates and expressed in the organizer region (embryonic shield in fish) at early gastrula stage. The impairment of ESC-E(Z) complex activity had no substantial effect on the level of *gsc* expression. In all of the morphants examined, the cells expressing *gsc* were located anterior to the *Pax2* stripe. Taking into account the severity of the cyclopia phenotype of *oleed* and *olezh2* morphants, it is not likely that impairment of nodal signaling was the main cause of this phenotype. In the second part, I tested the possibility that *hedgehog* signaling is impaired. At the 9-somite stage, normal extension of *shh* into the forebrain was not observed, whereas the expression in the mid/hindbrain and trunk region appeared unaltered. Notably, this lack of *shh* was only observed in the ventral forebrain, where *hedgehog* signaling is required for normal ventralization of the forebrain and act as an autoinductive signal. This result prompted us to postulate that *hedgehog* signaling is sufficiently perturbed in the morphants to cause cyclopia. To verify this possibility, I analyzed the expression of *hedgehog* target genes in MO injected embryos. It was shown that medaka *spalt* is a *hedgehog* target gene and that *Pax2* expression in the proximal part of the eye depends on *hedgehog* activity. I observed an extensive reduction of *spalt* expression in the ventral CNS and of *Pax2* expression in the optic stalk in the MO injected embryos as reported by Köster *et al*. (Köster *et al*. 1997). I then asked whether they act downstream of *hedgehog* signaling. Activation of *hedgehog* signaling by overexpressing *shh* results in expansion of *spalt* expression domains. Coinjection of MOs with *shh* mRNA suppressed the expansion of *spalt* domains, revealing the dependency of the expression of the *hedgehog* target gene on ESC-E(Z) complex. Taken together, I hypothesized that the activity ESC-E(Z) complex are required for *hedgehog* signaling activity

In the 6th chapter, I verify the above mentioned hypothesis. Based on the hypothesis, the activation of *hedgehog* signaling should lead to an increase in the activity of the ESC-E(Z) complex, which harbors an intrinsic activity for methylating histone H3 at lysine 27 (H3-K27). Accordingly, activation of *hedgehog* signaling might result in an increased amount of methylated histone. To determine whether this inference is correct, mouse NIH-3T3 cells were cultured with or without the Shh polypeptide and the extracted proteins were subjected to immunoblot analysis using a specific antibody for tri-methylated lysine 27 of histone H3 (tri me H3-K27). As expected, the level of methylated histone H3 was increased in Shh-treated cells (~2 fold), a finding that strongly supports the hypothesis. Additionally, the proteins of morphants were extracted and subjected to immunoblot analysis using the specific antibody for tri-methylated H3-K27. As expected, the level of tri-methylated H3-K27 was substantially decreased in both *olezh2* and *oleed* morphants. This reduction of tri-methylated H3-K27 in the morphants strongly suggests that the medaka ESC-E(Z) complex also tri-methylates H3-K27. Furthermore, the level of tri-methylated H3-K27 was estimated in *hedgehog* perturbed embryos. Treatment of medaka embryos by serially diluted forskolin to inhibit *hedgehog* signaling reduced the amount of tri-methylated H3-K27 in a dose-dependent fashion. This *in vivo*

evidence strongly supports the previous *in vitro* evidence with cultured cells. I assume that the cells that respond to the signaling utilize the activity of the ESC-E(Z) complex to fulfill its differentiation program.

In the 7th chapter, I verified the possibility that Six3 cooperates with ESC-E(Z) complex to respond to the *hedgehog* signaling. It has been reported that *Six3* is one of the responsible genes for cyclopia phenotype in human and medaka (Wallis et al., 1999, Carl et al., 2002), and that *Six3* confers cells the competence to respond to *hedgehog* signaling (Kobayashi et al., 2002). A report on medaka Six3 identified a cell-cycle regulator geminin as a binding partner of Six3 (Del Bene et al., 2004) and another work on mouse revealed that geminin interacts with one of the PcG proteins (Luo et al., 2004). In *oleed* and *olezh2* morphants, *Six3* expression was slightly reduced in retinal anlage at late neurula (st.18) and almost lost at 9-somite stage (st.22). It is interesting to note that the expression of this gene seems to reduce progressively. The same phenomenon was also observed in Six3 morphants itself (Carl et al., 2002). Based on this observation, it has been suggested that *Six3* acts in a regulatory feedback loop. I postulated that ESC-E(Z) complex activity is required for Six3 and that the expression of *Six3* is reduced progressively in our *olezh2* and *oleed* morphants. To confirm the cooperation of ESC-E(Z) complex and *Six3*, pairwise co-injection of *olezh2* MO and *oleed* MO with *Six3* MO was performed. The amount of each MO was reduced progressively until the frequency of the normal embryos reached more than 70%. The co-injection of the both *olezh2* MO and *Six3* MO as well as *oleed* MO and *Six3* MO resulted in the significant change in the ratio of cyclopia phenotype, which is indistinguishable from morphant phenotypes caused by higher amount of each MO alone. This synergistic effect observed by co-injection of low-dose MOs suggested that the ESC-E(Z) complex cooperates with Six3.

In the 8th chapter, I gave conclusions to this study. My findings provided the first evidence that chromatin modification such as histone lysine methylation is induced by *hedgehog* signaling. The N-terminal domains of histones are subject to various posttranslational modifications. Among them, histone lysine methylation is a very stable modification, suitable as a long-term molecular mark, and is expected to play an important role in the epigenetic inheritance of chromatin states. On the other hand, *hedgehog* proteins are known to be diffusible morphogens, which are involved in the induction and patterning processes in vertebrate and invertebrate embryos. An increasing body of evidence appears to support the scenario that the initial patterns set primarily by diffusible signals are subsequently translated into expression of a distinct set of transcription factors. Here, I provide an additional dimension to this scenario. The expression of a distinct set of transcription factors by these diffusible signals requires some maintenance activities such as histone lysine methylation, thereby enabling cells to achieve their destiny.

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